

Claims

What is claimed is:

- 5 1. A method for producing a polypeptide, comprising:
 - (a) cultivating a mutant of a parent filamentous fungal cell, wherein (i) the mutant comprises a first nucleic acid sequence encoding the polypeptide and a second nucleic acid sequence comprising a modification of at least one of the genes involved in the production of a trichothecene under conditions conducive for the production of the polypeptide, and (ii) the
10 mutant produces less of the trichothecene than the parent filamentous fungal cell when cultured under the same conditions; and
 - (b) isolating the polypeptide from the cultivation medium.
- 15 2. The method of claim 1, wherein the filamentous fungal cell is a an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Gibberella*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Myrothecium*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Piromyces*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, or *Trichoderma* strain.
- 20 3. The method of claim 1, wherein the filamentous fungal cell is a *Fusarium* strain.
4. The method of claim 3, wherein the *Fusarium* cell is a *Fusarium venenatum* cell.
5. The method of claim 4, wherein the *Fusarium venenatum* cell is *Fusarium venenatum*
25 ATCC 20334.
6. The method of claim 4, wherein the *Fusarium venenatum* cell is a morphological mutant.
- 30 7. The method of claim 6, wherein the *Fusarium venenatum* cell is a morphological mutant of *Fusarium venenatum* ATCC 20334.

8. The method of any of claims 1-7, wherein the gene is selected from the group consisting of *tri3*, *tri4*, *tri5*, *tri6*, *tril1*, *tril1*, *tril2*, and *tri101*.

5 9. The method of any of claims 1-8, wherein the gene is a *tri3* or trichodiene synthase gene.

10. The method of any of claims 1-9, wherein the gene is a *tri3* gene.

10 11. The method of any of claims 1-10, wherein the gene is a *tri4* gene.

12. The method of any of claims 1-11, wherein the gene is a *tri6* gene.

13. The method of any of claims 1-12, wherein the gene is a *tril1* gene.

15 14. The method of any of claims 1-13, wherein the gene is a *tril2* gene.

15. The method of any of claims 1-14, wherein the gene is a *tri101* gene.

20 16. The method of any of claims 1-15, wherein the mutant cell produces at least about 25% less of the trichothecene than the parent filamentous fungal cell when cultured under identical conditions.

17. The method of any of claims 1-16, wherein the mutant cell produces no trichothecene.

25 18. The method of any of claims 1-17, wherein the filamentous fungal cell comprises at least two copies of the first nucleic acid sequence.

30 19. The method of any of claims 1-18, wherein the heterologous polypeptide is a hormone, a hormone variant, an enzyme, a receptor or a portion thereof, an antibody or a portion thereof, or a reporter.

20. The method of claim 19, wherein the enzyme is an oxidoreductase, a transferase, a

~~hydrolase, a lyase, an isomerase, or a ligase.~~

21. The method of claim 20, wherein the enzyme is an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, a pectinolytic enzyme, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, or xylanase.

22. The method of any of claims 1-21, wherein the mutant cell further comprises one or more modifications of one or more third nucleic acid sequences, wherein the modification reduces or eliminates expression of the one or more third nucleic acid sequences.

23. The method of claim 22, wherein the one or more third nucleic acid sequences independently encode an enzyme selected from the group consisting of an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, a pectinolytic enzyme, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, and xylanase.

24. The method of any of claims 22, wherein the one or more third nucleic acid sequences independently encode an aminopeptidase, carboxypeptidase, or protease.

25. The method of any of claims 1-24, wherein the second nucleic acid sequence comprising a modification of at least one of the genes involved in the production of a trichothecene is not marked with a selectable marker.

26. A trichothecene-deficient mutant cell of a filamentous fungal cell, comprising a first nucleic acid sequence encoding a heterologous polypeptide and a second nucleic acid sequence comprising a modification of at least one of the genes responsible for the production of a trichothecene, wherein the mutant produces less of the trichothecene than the

~~parent filamentous fungal cell of the mutant cell when cultured under the same conditions.~~

27. The mutant cell of claim 26, wherein the filamentous fungal cell is a an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Gibberella*, *Humicola*,
5 *Magnaporthe*, *Mucor*, *Myceliophthora*, *Myrothecium*, *Neocallimastix*, *Neurospora*,
Paecilomyces, *Penicillium*, *Piromyces*, *Schizophyllum*, *Talaromyces*, *Thermoascus*,
Thielavia, *Tolypocladium*, or *Trichoderma* strain.

28. The mutant cell of claim 26, wherein the filamentous fungal cell is a *Fusarium* strain.

29. The mutant cell of claim 28, wherein the *Fusarium* cell is a *Fusarium venenatum* cell.

30. The mutant cell of claim 29, wherein the *Fusarium venenatum* cell is *Fusarium venenatum* ATCC 20334.

31. The mutant cell of claim 29, wherein the *Fusarium venenatum* cell is a morphological mutant.

32. The mutant cell of claim 31, wherein the *Fusarium venenatum* cell is a morphological mutant of *Fusarium venenatum* ATCC 20334.

33. The mutant cell of any of claims 26-32, wherein the gene is selected from the group consisting of *tri3*, *tri4*, *tri5*, *tri6*, *tril1*, *tril1*, *tril2*, and *tri101*.

34. The mutant cell of any of claims 26-33, wherein the gene is a *tri5* or trichodiene synthase gene.

35. The mutant cell of any of claims 26-34, wherein the gene is a *tri3* gene.

36. The mutant cell of any of claims 26-35, wherein the gene is a *tri4* gene.

37. The mutant cell of any of claims 26-36, wherein the gene is a *tri6* gene.

38. ~~The mutant cell of any of claims 26-37, wherein the gene is a *tril1* gene.~~

39. The mutant cell of any of claims 26-38, wherein the gene is a *tril2* gene.

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40. The mutant cell of any of claims 26-39, wherein the gene is a *tril01* gene.

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41. The mutant cell of any of claims 26-40, wherein the mutant cell produces at least about 25% less of the trichothecene than the parent filamentous fungal cell when cultured under identical conditions.

42. The mutant cell of any of claims 26-41, wherein the mutant cell produces no trichothecene.

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43. The mutant cell of any of claims 26-42, wherein the filamentous fungal cell comprises at least two copies of the first nucleic acid sequence.

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44. The mutant cell of any of claims 26-43, wherein the heterologous polypeptide is a hormone, a hormone variant, an enzyme, a receptor or a portion thereof, an antibody or a portion thereof, or a reporter.

45. The mutant cell of claim 44, wherein the enzyme is an oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase, or a ligase.

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46. The mutant cell of claim 45, wherein the enzyme is an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, a pectinolytic enzyme, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, or xylanase.

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47. The mutant cell of any of claims 26-46, wherein the mutant cell further comprises one

or more modifications of one or more third nucleic acid sequences, wherein the modification reduces or eliminates expression of the one or more third nucleic acid sequences.

48. The mutant cell of claim 27, wherein the one or more third nucleic acid sequences independently encode an enzyme selected from the group consisting of an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase, lipase, mannosidase, mutanase, oxidase, a pectinolytic enzyme, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase, and xylanase.

49. The mutant cell of claim 47, wherein the one or more third nucleic acid sequences independently encode an aminopeptidase, carboxypeptidase, or protease.

50. The mutant cell of any of claims 26-49, wherein the second nucleic acid sequence comprising a modification of at least one of the genes involved in the production of a trichothecene is not marked with a selectable marker.

51. A method for obtaining the mutant cell of any of claims 26-50, comprising:

(a) introducing into a parent filamentous fungal cell a nucleic acid sequence comprising a modification of at least one of the genes responsible for the production of a trichothecene; and

(b) identifying the mutant from step (a), wherein the mutant produces less of the trichothecene than the parent filamentous fungal cell of the mutant cell when cultured under the same conditions.

52. An isolated trichodiene synthase obtained from a *Fusarium venenatum* strain, selected from the group consisting of:

(a) a trichodiene synthase having an amino acid sequence which has at least 97% identity with SEQ ID NO. 2;

(b) a variant of the trichodiene synthase having an amino acid sequence of SEQ ID NO. 2 comprising a substitution, deletion, and/or insertion of one or more amino acids;

- ~~(c) an allelic variant of (a) or (b); and~~
~~(d) a fragment of (a) or (c) that has trichodiene synthase activity.~~

53. The trichodiene synthase of claim 52, having an amino acid sequence which has at
5 least 97% identity with SEQ ID NO. 2.

54. The trichodiene synthase of claim 52, comprising the amino acid sequence of SEQ ID
NO. 2 or a fragment thereof.

10 55. The trichodiene synthase of claim 52, comprising the amino acid sequence of SEQ ID
NO. 2.

56. The trichodiene synthase of any of claims 52-55, which is obtained from *Fusarium*
venenatum ATCC 20334.

15 57. The trichodiene synthase of claim 52, which is encoded by the nucleic acid sequence
contained in plasmid pTri5 contained in *E. coli* NRRL B-30029.

58. An isolated nucleic acid sequence comprising a nucleic acid sequence which encodes
20 the trichodiene synthase of any of claims 52-57.

59. A nucleic acid construct comprising the nucleic acid sequence of claim 58 operably
linked to one or more control sequences which direct the production of the trichodiene
synthase in a suitable expression host.

25 60. A recombinant expression vector comprising the nucleic acid construct of claim 59.

61. A recombinant host cell comprising the nucleic acid construct of claim 59.

30 62. A method for producing the trichodiene synthase of any of claims 52-57 comprising
(a) cultivating a *Fusarium venenatum* strain to produce a supernatant comprising the
trichodiene synthase; and (b) recovering the trichodiene synthase.

63. A method for producing the trichodiene synthase comprising (a) cultivating the host cell of claim 61 under conditions suitable for production of the trichodiene synthase, and (b) recovering the trichodiene synthase.

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64. A method for obtaining a mutant cell, comprising:

(a) introducing into a parent cell, having a first nucleic acid sequence encoding a first gene product, a first nucleic acid construct comprising a nitrate reductase gene as a selectable marker and a modification of the first nucleic acid sequence, wherein the first construct incorporates into the genome of the parent cell replacing the endogenous first nucleic acid sequence with the modified first nucleic acid sequence resulting in reduced production of the first gene product compared to the parent cell when cultivated under the same conditions; and

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(b) selecting a mutant cell from step (a) for the presence of the nitrate reductase gene and reduced production of the first gene product.

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65. The method of claim 64, further comprising:

(c) selecting a mutant cell from step (b) under culturing conditions in which the nitrate reductase gene is deleted.

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66. The method of claim 64, further comprising:

(c) introducing into the mutant cell from step (b) a second nucleic acid construct comprising a second nucleic acid sequence comprising 5' and 3' regions of the modified first nucleic acid sequence, but lacking the nitrate reductase gene, wherein the second construct incorporates into the genome of the parent cell replacing the modified first nucleic acid sequence with the second nucleic acid sequence; and

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(d) selecting a mutant cell from step (c) under culturing conditions in which the nitrate reductase gene is deleted.

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67. A mutant cell obtained by the method of any of claims 64-66.

68. A method for producing a polypeptide, comprising:

- (a) cultivating the mutant cell of claim 67 comprising a nucleic acid sequence encoding a polypeptide under conditions conducive for the production of the polypeptide; and
- (b) isolating the polypeptide from the cultivation medium of the mutant cell.

5 69. A method for introducing an alteration into a target nucleic acid sequence contained in a cell, comprising:

- (a) obtaining mutant cells of a parent cell by introducing into the parent cell a nucleic acid construct comprising a nitrate reductase selectable marker gene, flanked by nucleotide sequence repeats to form a marker cassette, wherein the repeats consist of a
- 10 nucleotide sequence immediately 5' or a nucleotide sequence immediately 3' of the target DNA; and further wherein

when the flanking nucleotide sequence repeats consist of a nucleotide sequence 5' of the target DNA, the nucleic acid construct further comprises, 3' of, and contiguous to, the marker cassette, an additional nucleotide sequence consisting of a

15 nucleotide sequence 3' of the target DNA sequence; and wherein

when the flanking nucleotide sequence repeats consist of a nucleotide sequence 3' of the target DNA, the nucleic acid construct further comprises, 5' of, and contiguous to, the marker cassette, an additional nucleotide sequence consisting of a nucleotide sequence 5' of the target DNA sequence;

- 20 (b) selecting mutant cells from step (a) by selecting for the presence of the nitrate reductase gene;

(c) cultivating the selected mutant cells under conditions effecting deletion of the nitrate reductase gene by recombination between the flanking nucleotide sequence repeats; and

- 25 (d) selecting a mutant cell with an alteration in the target nucleic acid sequence in which the nitrate reductase gene has been deleted from the mutant cell.

70. A mutant cell obtained by the method of claim 69.

30 71. A method for producing a polypeptide, comprising:

- (a) cultivating the mutant cell of claim 70 comprising a nucleic acid sequence encoding a polypeptide under conditions conducive for the production of the polypeptide; and

- (b) isolating the polypeptide from the cultivation medium of the mutant cell.

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